STUDIES ON SPERMATHECAL FILLING IN AEDES AEGYPTI (LINNAEUS). II. EXPERIMENTAL[1]

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Our knowledge of the physiology of sperm transport in the vertebrates is very extensive (see, *e.g.*, Parkes, 1960, and Bishop, 1961), but relatively little experimental information is available for insects. Most of what is known about sperm transport in insects has been well reviewed by Wigglesworth (1953) and Hinton (1963). It is generally believed that the female is largely or entirely responsible for translocating sperm to her storage organs in many Hemiptera (Davey, 1958), Coleoptera (Blunck, 1912), Lepidoptera (Hewer, 1934; Omura, 1938; Callahan and Cascia, 1963), and Hymenoptera (Ruttner, 1956; Snodgrass, 1956). In some Hemiptera, the sperm are said to be injected by the male directly into the spermathecae (Ludwig, 1926; Bonhag and Wick, 1953). In certain insects chemotactic substances within the female are claimed to be important in sperm transport (*e.g.*, Weidner, 1934). On the other hand, in *Drosophila*, it has been suggested that the sperm are largely or entirely responsible for their transport within the female (Nonidez, 1920). In *Anopheles* mosquitoes, Giglioli (1963) stated (p. 166) that ". . . ejaculation appears to be the direct passage of spermatozoa through the phallotreme into the spermathecal duct . . . ," and he suggested (p. 160) that sperm transport could be ". . . best explained by the existence of a partial vacuum in the spermatheca during insemination; this being achieved by peristaltic contractions of the . . . [spermathecal] duct." Descriptions of spermathecal filling in *Aedes aegypti* are given by Burcham (1957a), Schwartz (1961), Spielman (1964), and by Jones and Wheeler (1965).

In the present study, an attempt was made to identify the major factors involved in the filling of the spermathecae after insemination of the Bangkok strain of the yellow fever mosquito. Specifically, it was our aim to determine whether spermatozoa locomote within a passive female reproductive tract, whether some action of the female is solely responsible for their translocation, or whether both motility of the sperm and the female's activity are required to fill the spermathecae. We have by no means resolved the question, but this paper sets forth the problems that must be solved before we shall have a very clear understanding of sperm transport in aedine mosquitoes.

Methods

The mosquitoes were reared as previously described (Jones and Wheeler, 1965). In order to manipulate the mosquitoes, they were first anesthetized with

[1] This research was sponsored by N. I. H. Grant GM 06021 and by Development Award 1 K-3-GM 21,529 to the first author. Scientific Article Number A 1201, Contribution Number 3699 of the Maryland Agricultural Experiment Station.

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nitrogen. Two techniques were used to study sperm transport. The first one involved rubbing the terminalium of the male against that of the female at an angle of about 90° under a dissecting stereo-microscope at a magnification of 13 ×. The dorsal surface of the thorax of each mosquito had previously been glued to the head of a separate pin and the manipulations were made using Wheeler's apparatus (1962). Basically, this is the technique of McDaniel and Horsfall (1957) and will be referred to hereafter as *forced-copulation* or *forced-mating*. The second method involved injection of the bursae of virgin females with a variety of materials, using a fine glass micropipette and the assembly described by Wheeler and Jones (1963). The great majority of the artificial inseminations were made using *Drosophila* Ringer (Ephrussi and Beadle, 1936) buffered to pH 6.8. The microscope cold-stage used in some of the experiments was that described by Wheeler (1964). For other details see the text.

Results

1. *Effect of the female's position on spermathecal filling*

Immediately following free mating, *Aedes* females perch themselves so that their abdominal tip faces down. To determine whether shifting the normal posture of the freshly inseminated female would affect spermathecal filling, immediately after forced-copulation, females were oriented into the following positions: abdominal tip facing (a) up, (b) down, (c) to the side, or (d) the female was turned upside down. Four to 9 females were used for each position. In each case two to three thecae filled with sperm, and the results in no way differed from those of females which had been force-copulated and released into a cage. Thus, spermathecal filling is not affected by the position of the female.

2. *Effect of certain surgical operations on spermathecal filling*

In one experiment the heads of 6 virgins (4–7 days old) were crushed with forceps before presenting them to unmated males for forced-copulation. Only three of the females force-copulated. Of these three, one was not inseminated. One had ejaculate on the outside of her genital lips and apparently some within her vagina, but no sperm were seen in the bursa or thecae. However, the third female had been properly inseminated: the bursa was fully distended with ejaculate and two of her thecae contained numerous sperm 64 minutes after coitus. Five control females all readily force-copulated and transferred sperm to the thecae.

The experiment was repeated using 17-day-old mosquitoes. Of the 6 females used, one did not copulate with any of three potent males. One female did not accept the first male but did accept the second; this copulation lasted 350 seconds, but the female was not inseminated. The other four females all force-copulated with the first male presented, and received seminal material, but only three of them had few to many sperm in two thecae.

Five 3-to-5-day-old virgin females were decapitated and all 5 of them force-copulated with previously unmated males (they copulated for 6 to 43 seconds, with a mean of 17.6 seconds).

Ten 15-day-old virgin females were decapitated and all 10 force-copulated with 15-day-old males. Most copulated for an abnormally long time (21 to 242 seconds,

with a mean of 91.2), but only 6 were inseminated and only four had few to many sperm in two thecae.

These experiments indicate that crushing the head or decapitation of the female prior to mating may lead to erratic findings with forced-copulation.

Heads of four females were crushed during the act of forced-copulation (*i.e.*, as soon as the male had made full genital contact), and the females were dissected in 5 to 12 minutes. All four females had been inseminated and all had sperm in two to three thecae. Heads of 5 females were crushed immediately after withdrawal of the male, and the females were dissected in 16 to 17 minutes. One of the females had not been inseminated, but the other four females had sperm in two to three thecae. Thus, damaging the head of the female during or immediately after forced-coitus in no way interferes with spermathecal filling.

Five females were force-copulated for 15 seconds and within 30 seconds, or less, their terminalia cut off and placed in an open drop of saline on a glass slide. Four minutes later the thecae were dissected. Four of the 5 females had been inseminated. Of the 15 thecae examined, three had many sperm, two had few to many sperm, three had very few sperm, and 7 had no detectable sperm. The results did not obviously differ from controls. Thus, spermathecal filling is controlled by forces within the terminalium alone.

On four occasions, where the intact bursa, vagina and spermathecal complex had been isolated into a drop of saline and covered with a layer of immersion oil within less than 30 seconds after mating, we observed the empty thecae suddenly fill with many spermatozoa. In one case the sperm did not fully enter the thecae but vibrated within the base of the large theca like a sheaf of delicate spears. In another case, many sperm shot suddenly into the median theca and quickly began to spin around within it. In two other cases, two thecae completely filled with sperm. In all four cases, the thecae filled within the first 45 seconds of the observations and very rapidly stopped. Unfortunately, the spermathecal ducts were not clearly visible in any of these preparations, but the bursal orifice and vestibule were visible in two cases and the sperm were exceedingly active in these regions. Thus, spermathecal filling can occur *in vitro* and the forces responsible for it are located in a highly specific portion of the female reproductive tract.

Seven virgin females which had fed on blood three days previously were force-copulated for 15 seconds and immediately thereafter were decapitated to induce rapid egg deposition. Five of these females began to lay eggs. The ovipositing females were dissected within 5 to 10 minutes. All five of them had two thecae filled with numerous sperm. This is not proof, however, that the thecae filled with sperm during egg deposition because the females were dissected after the sperm are known to be able to reach the thecae and the sperm could have easily ascended during the intervals between egg depositions. The eggs did not hatch. This technique is currently being used to study the important question of sperm capacitance.

3. *Effects of exposure to nitrogen, ether, carbon dioxide and cyclopropane on spermathecal filling*

Although nitrogen quickly immobilizes *A. aegypti,* they recover quite rapidly when placed in air. Five females were force-copulated for 15 seconds and placed in

a steady stream of nitrogen. After being hosed with nitrogen for four minutes, they were dissected in about 45 seconds. All 5 females had sperm in two thecae. Of the 15 thecae examined one had many sperm, 5 had few to many sperm, four had very few sperm, and 5 had no sperm. Identical results were obtained with 5 controls kept in air for four minutes.

Three females were placed in ether vapors for 15 to 30 seconds and afterwards quickly force-mated for 10 to 21 seconds. The females recovered in less than one minute and were dissected after 5 to 10 minutes. Two of the females had been inseminated, and both of these had active sperm in their distended bursae and few to many sperm in one to two thecae. Four females were force-mated for about 17 seconds and then quickly etherized for one to two minutes. They were allowed to stand in air for 5 minutes before dissection. All of the females had few to many sperm in two thecae. Ten females were force-mated for 10 to 15 seconds, allowed to stand in air to 30 to 90 seconds, then placed in strong ether vapors until dead and were dissected. All of the females had their bursae distended with ejaculate, and all had few to many sperm in one to two thecae. Five females were force-copulated for 10 to 15 seconds, allowed to stand in air for one minute, then placed in ether vapors for one to five minutes, and then dissected. All females had few to many sperm in one to two thecae. In three of the females, the thecal sperm were active.

Five females were forced-mated for 15 seconds and placed in a stream of carbon dioxide for four minutes and dissected within one minute. Four out of the 5 females had few to many sperm in one to two thecae; the sperm were very intensely active in both the bursae and thecae. Of the 15 thecae examined, one had many sperm, three had few to many sperm, 5 had very few sperm, and 6 thecae had no sperm. The results did not obviously differ from controls, except that carbon dioxide seems to increase sperm activity.

Five females were force-copulated for 15 seconds, and in one to three seconds were placed in a stream of cyclopropane where they were quickly immobilized. They were hosed for 5 minutes and dissected in 28 to 51 seconds. Four of the females had been inseminated, and each of them had sperm in two thecae. Of the 15 thecae studied, two had numerous sperm, three had few to many sperm, three had very few sperm, and 7 thecae had no sperm. The results did not differ from controls.

Thus, a variety of gaseous anesthetics which externally immobilize the female do not necessarily affect spermathecal filling.

4. *Effects of chilling on sperm and spermathecal filling*

Ten females were force-copulated and within 5 seconds were completely submerged in an ice-water bath at about 7° C. for 10 to 20 minutes. The females were removed and dissected in ice-cold saline within approximately 30 seconds. One of the females had not been inseminated. Nine of the females had distended bursae with very active sperm. Of these 9 females, four had no sperm in the thecae. But 5 out of the 9 females had sperm in two thecae. Three females were force-mated and placed in an ice bath at 4° C. for 10 to 30 minutes. One of the females had not been inseminated. The other two females had the bursa with

ejaculate containing active sperm, and in both of these cases a few sperm had reached the thecae. Five virgins were forced-copulated for 15 seconds, and in 8 to 10 seconds they were totally submerged in an ice-water bath at 1–2° C. for 30 minutes. They were dissected at room temperature within 32 to 41 seconds. Four of the females had sperm in two thecae, and one female had sperm in all three thecae. Of the 15 thecae examined, three contained numerous sperm, three contained many sperm, three contained few to many sperm, two contained only a few sperm, and four thecae had no sperm. Since it was possible that sperm could have started to reach the thecae during the seconds it required to dissect them, another experiment was made in which the females were dissected into a drop of cold saline on a special microscope cold-stage held at 1–5° C. Four females were force-copulated for 10 seconds, and in about 5 to 10 seconds they were submerged in an ice-water bath at 1–2° C. for 18 to 67 minutes. One of these females had a greatly distended bursa with numerous, dense, circular clusters of inactive sperm. The bursal wall was vacuolated and swollen, but the ejaculate was not vacuolated. No sperm were in the thecae of this female. In the second female the bursa was fully distended, and sperm were active at the bursal orifice but only a few active sperm were visible in the large theca and no sperm were in the lateral thecae; the ejaculate was not vacuolated. In the third female, the bursa was distended with non-vacuolated ejaculate, and a few sperm were active at the bursal orifice, but only a few sperm were present in two thecae. The fourth female was removed from the ice bath after 67 minutes, held at room temperature (27° C.) for about one minute, and then dissected. Many active sperm were present in two thecae. The ejaculate did not vacuolate during the 10-minute observation period.

To determine whether certain organs of the female would remain active at low temperatures, the hind gut and reproductive system were dissected into a drop of cold saline on the special microscope cold-stage held at 4° C. In one case, the hind gut and reproductive system did not contract during the 10-minute observation period. When the temperature was allowed to rise to about 7° C., the hind gut very slowly contracted; but the ovaries and lateral oviducts did not contract. In another case, held at 3 to 4° C., the hind gut and lateral oviducts very slowly contracted, but the ovaries were inactive. In a third case, the vagina and lateral oviducts did not contract at 1° to 4° C.

To determine whether sperm would remain active at low temperatures, the male reproductive system was isolated in an open drop of saline on a cold-stage held at 5° to 6° C. The sperm remained quite active in the posterior chamber of both testes for at least one hour and in the sperm ducts for about 30 minutes, but the sperm in the seminal vesicles were inactive. Sperm released into the saline remained actively undulating for about 6 minutes; those sperm within the vicinity of the ruptured seminal vesicles, however, remained active for about 30 minutes. At 1° to 2° C. the activity of the sperm seemed definitely greatly reduced or abolished.

These experiments indicate that spermathecal filling can occur at 4° to 7° C. at which temperatures the contractions of the female reproductive system are greatly reduced or abolished. At these same temperatures, however, the sperm remain quite active. At 1° to 2° C. spermathecal filling tended to be greatly reduced, the ejaculate in the bursa did not vacuolate, and the sperm were mostly

inactive. That the sperm of *Aedes* can remain active for some time at 5° to 6° C. is in marked contrast to the sperm of *Periplaneta* which are mostly inactive at 10° C. (Richards, 1963). The sperm of the honey bee are reported to survive two hours "contact with ice" (Bishop, 1920).

5. *Effect of killing the female just prior to forced copulation on spermathecal filling*

Before being force-copulated, females were killed by overexposure to cyanide vapors (three cases), carbon dioxide (two cases), ether vapors (14 cases), or cold temperatures (7 cases). Of the 26 females used, two were unacceptable to a series of previously unmated and highly potent males. Of the 24 remaining females, 7 did not receive ejaculate. Males ejaculated on the outside of the genitalia of 8 (47.6%) of the 17 dead females. However, males deposited ejaculate in the bursae of 9 of the dead females. The sperm were inactive in the bursae of two of these. However, in 7 cases, the bursae of the dead females contained active spermatozoa. No sperm were seen in the thecae of these dead females at 10, 20, or 30 minutes after copulation. In most of the cases studied, the females were not severely dehydrated and the genital tract was generally not conspicuously blocked or otherwise distorted. However, overexposure to carbon dioxide can greatly distort the female's genital system (Spielman, personal communication) in such a way as to make sperm migration impossible.

6. *Distribution of dyes after injection into the bursa*

Using the apparatus of Wheeler and Jones (1963), various dyes in saline, with and without certain additives, were injected into the bursae of 114 virgins and the females dissected at varying intervals to determine the location of the dye. The data in Table I are based only on injected mosquitoes which appeared essentially normal.

As shown in Table I, (1) certain dyes injected only into the bursae of virgins are capable of being transported into the common oviduct, as far as the ampullae, and into one, two or all three thecae; (2) dyes may be detectable within the thecae without being visible within the thecal ducts; (3) the occasional presence of dyes in the periductal glands and in the basal glands of the spermathecae indicates that some of the dyes may be absorbed from the bursa into the hemolymph; (4) the addition of a variety of organic substances to the dye solutions did not seem to enhance the degree or extent of dye transport. In these studies, dyes were never found in the female's accessory gland or its duct. In all of the cases of bursal injection with dye, the bursal wall did not swell or develop vacuoles.

Some of the data in Table I need amplification. Four virgins whose bursae had been injected with congo red were force-copulated 16 to 21 minutes later, and the females dissected after standing for 20 to 24 minutes. The first female was not inseminated. The second mosquito had sperm in two thecae, but no dye was seen in any of her thecae. In the third case, sperm were in all three thecae but dye was questionably present only in the large median theca. In the fourth female, sperm were in all three thecae but dye was detectable in only two. No dye was seen in any of the spermathecal ducts.

The bursae of 5 virgins injected with congo red saline were force-copulated 20 hours later and dissected after 25 to 30 minutes. In the first female, sperm were in two thecae, but dye was detectable only in the bursa and common oviduct. In the second mosquito, the large theca was bright red, but no sperm were found in any of her thecae; very few sperm were visible in the bursa. In the third case, two

TABLE I

Location of dyes within the female genital tract of Aedes aegypti after injection into the bursa. SD = spermathecal ducts; PGD = periductal glands

Solution injected	No. ♀♀	Time dissected	Location of dye (no. cases)						
			Bursa	Oviduct	SD	PDG	Spermathecae		
							Median	Lateral 1	Lateral 2
Methylene blue	1	15 min.	1	1	0	0	0	0	0
Amaranth red	5	32-109 min.	5	3	0	0	2	2	1
	12	24 hrs.	12	3	1	7	2	0	0
+sonofied milk	10	24 hrs.	10	2	4	4	0	1?	0
+ glycogen	15	19-24 hrs.	15	1	3	12	3;1?	0	0
+ 10^{-4}% noradrenalin	8	20 hrs.	8	0	2	4	0	0	0
+ 2% egg albumin	3	20 hrs.	3	0	0	0	0	0	0
+ extract ♂ reprod. system	3	19 hrs.	3	0	2	2	1	2	2
+ extract 20 ♂ acc. glands	5	22 hrs.	5	1	3	3	3	1	0
Sudan red in Shillaber's oil	1	10 min.	1	0	0	0	0	0	0
Congo red	7	20–59 min.	7	4	2	1	1	0	0
	5	32–109 min.	5	3	0	0	2	2	1
	10	115-150 min.	10	5	1	0	2	2	1
	5	20 hrs.	5	3	0	0*	1;1?	0	0
+ fructose	5	96–100 min.	5	1	0	0	1	0	0
+ force cop. after 16–21 minutes	4	20–24 min.	4**	0	0	0	2;1?	1;1?	0
+ force cop. after 20 hrs.	5	25–30 min.	5**	3	0	0$^{\phi}$	4†	1	0
Cage cop. 3 hrs; then inject bursa with congo red	10	90–117 min.	10	5	1	0	1‡	1‡	1‡

* Orange in basal glands of spermathecae.

** Ejaculate in three females' bursae.

ϕ Dye in basal glands of three females.

† Dye in one theca of both uninseminated females.

‡ Dye in all three thecae of the one uninseminated female.

thecae had sperm, but dye was clearly present in all three thecae; her common oviduct also had dye in it. Two of the mosquitoes had not been inseminated; and in both of them, dye was present in the large median theca only. Dye in those thecae which did not contain sperm probably was transported to them shortly after injection into the bursae.

Two to three hours after free-copulation, the bursae of 10 females were injected with congo red saline and the mosquitoes were dissected in 90 to 117 minutes. Nine of them had been inseminated. Dye was present in all bursae and in the common oviduct of 5. Dye was visible in all three thecae of the one uninseminated female and *not* visible in any of the inseminated females' thecae. This suggests that dye transport to the thecae ceases after the thecae fill with sperm.

How the dyes become distributed to the thecae is not clear. Whatever the explanation, it seems evident that a mechanism exists within the virgin female reproductive system which could direct sperm into the thecae, and this mechanism stops when the thecae fill with sperm.

7. Distribution of various sperm mixtures after injection into the bursa

The bursae of 94 females were successfully injected with many to very numerous usually highly motile sperm under a variety of experimental conditions. The females were not roughly handled before, during or after artificial insemination, and although some injury may have occurred, it is considered minimal for the cases reported here. The data may be summarized in the following statements. (1) Pure undiluted seminal vesicle sperm did not reach the thecae within 84 minutes (one case). (2) In one out of 6 cases, after injection of seminal vesicle sperm in saline into the bursa, only a very few sperm reached one theca in 10 to 150 minutes. (3) A very few sperm reached the large theca in two out of 8 cases within 19 to 24 hours after injection of the bursa with seminal vesicle sperm in amaranth red saline. (4) When noradrenalin ($10^{-4}\%$) and extracts of either the testes or of the whole male reproductive system were added to seminal vesicle sperm in saline, no sperm reached the thecae (10 cases). (5) In two out of 10 cases injected with seminal vesicle sperm in saline plus male accessory gland exudate, very few sperm reached only the large theca. When male accessory glands were ruptured in open saline drops, the exudate rapidly gelled, but when the glands were ruptured in saline under a layer of immersion oil, it was possible to withdraw exudate in suitable amounts.[3] (6) When seminal vesicle sperm were freshly collected into (a) fresh human seminal fluid (two cases), (b) sonofied sweet milk (four cases), (c) emulsified Shillaber's immersion oil (three cases), (d) testicular extract (one case), (e) fresh bull seminal fluid (one case), (f) fresh bursal ejaculate (three cases), and then injected into the bursae of virgin females, no sperm reached the thecae. (7) When fresh seminal vesicle sperm had been injected into the bursae and the females then quickly force-copulated with a sexually depleted male (*i.e.,* with a repetitively force-mated specimen) for one to 25 seconds, in only one out of 6 cases did a very few sperm reach the large theca. (8) Even when fresh ejaculate containing active sperm was quickly removed from the bursa of a freshly force-copulated donor female and this quickly injected into a recipient virgin bursa, sperm did not reach the thecae (three cases). (9) When seminal vesicle sperm were added to fresh ejaculates taken from a donor bursa and this injected into a virgin bursa and these recipient females then quickly force-copulated with a potent male for one to two seconds (*i.e.,* before the male could ejaculate), in three out of 5

[3] Burcham (1957a, 1957b) apparently found no difficulty in micropipetting male accessory gland exudate from open saline drops, but our micropipettes consistently clogged.

cases only a few sperm were found in one to two thecae. (10) When seminal vesicle sperm were collected into a small drop of saline which was covered with a layer of immersion oil prior to injection into the virgin bursa, better results were obtained in one exceptional series of animals in one experiment. Thus, in 7 out of 17 cases in which fresh seminal vesicle sperm were collected in saline under a layer of oil, few to many sperm reached the large theca. (11) Testicular sperm collected in saline under oil did not reach the thecae (three cases). (12) Attempts to inject spermathecal sperm (4 to 9 thecae were needed for a single injection) were not satisfactory (two cases). (13) In one experiment fresh ejaculate was quickly removed from the bursa of a donor female into a small drop of saline and covered with a layer of immersion oil. In two out of three cases, many sperm reached one to two thecae. (14) Mammalian sperm from fresh human (two cases) and fresh bull ejaculates (four cases) placed in the bursae were very numerous, highly active, and small enough to pass into the spermathecal ducts, yet none left the bursa and none reached the thecae. In several cases, the alien sperm heads were oriented away from the bursal orifice.

Thus, while sperm withdrawn from the seminal vesicles or from a donor bursa and artificially injected into a virgin bursa are capable of reaching the thecae in about 25% of the cases, the extent and degree of thecal filling are very low and far inferior to that following either forced-mating or free-mating. Attempts to increase the extent of filling with artificially injected sperm were generally unsuccessful.

In many of the cases where sperm did not reach the thecae, the bursa was greatly distended with the injected material; and the sperm within the bursa were often very numerous, highly motile, oriented, and exhibited violent activity near the bursal orifice. In several cases, the sperm injected within the bursa locomoted violently around within the sac.

8. *Results of artificial insemination*

A small amount of information was collected on the results of artificial insemination of *A. aegypti*. Ten females were injected with fresh bursal ejaculate collected under oil, then released into a cage and the females given sugar water and offered a blood meal. Ten days after being injected, 6 females were dead and one was missing. The three remaining females laid 50 eggs, none of which hatched. When the females were dissected, there were no sperm in their thecae.

Thirteen 7-day-old virgins were artificially inseminated with fresh seminal vesicle sperm collected in saline under oil. The donating males were 21 days old and had not been mated. Three females were dissected in 10 to 20 minutes, and all three of them had few to many sperm in two thecae. The other 10 females were released into a cage. The next day, one female was dead. The remaining 9 females were offered a blood meal, which 5 of them took. Three to four days thereafter, a batch of 18 eggs was found. All of these eggs hatched and subsequently developed into fourth stage larvae. Inadvertently, these larvae were killed due to gross overfeeding. Fifteen days after being artificially inseminated, four females were dissected for study. Two of them had sperm in the thecae; one female had not been inseminated, and one female had inactive sperm in her bursa and no sperm in her thecae. The 5 remaining females were offered a second blood meal, and a batch of

8 eggs was subsequently found, all of which hatched and developed into active adult males and females.

Discussion

To account fully for spermathecal filling in the *Aedes* mosquito, one must explain how many spermatozoa leave a non-muscular sac, make a sharp U-turn, ascend highly convoluted but non-contracting ducts, and quickly enter fluid-filled spherical reservoirs which have no muscles.

There are several conceivable ways in which the female might play some role in sperm transport. First, the bursal wall might secrete a substance into the ejaculate which would be necessary for or useful to transport. There is no doubt that the bursal wall appears secretory after insemination, but this secretion appears to occur after sperm transport has already begun. Second, the female accessory gland might very briefly secrete a substance that would attract the sperm into the vestibule for only a short time. The female accessory gland is certainly well-situated for such a possibility. It is difficult to believe that the female accessory gland produces a chemically attractive substance, since seminal vesicle sperm fail to congregate about the gland or its orifice in *in vitro* preparations. Furthermore, there are no obvious histological differences in the secretory appearance of the female accessory gland before, during or after sperm transport. Presumably the gland is continuously secretory. Third, the fluid in the spermathecae or their ducts might have some chemically attractive substance occurring in a steep gradient. It seems improbable that the spermathecae have such a chemically attractive material because the sperm fail to congregate around the ducts in *in vitro* preparations and they do not specifically move towards crushed spermathecae in fresh whole-mounts. Fourth, the female might materially aid sperm transport by creating a current within the vagina. A current within this region could be produced by the brief rapid contractions of the transverse muscles of the spermathecal eminence as observed by Leahy (1962). Although such contractions might be elicited as a result of stimulation from the aedeagus during the act of coitus, we have not consistently seen such contractions immediately following forced-coitus in fresh dissections. An anteriorly-directed current is produced in the female genital tract by the vigorous rhythmic contractions of the lateral oviducts and ovaries (Curtin and Jones, 1961). This current might be regulated by closing or opening of the vaginal valves and/or the gonopore. Conceivably, an anteriorly-directed current within the vagina would help draw sperm out of the bursa into the upper vagina. Contractions of the muscles of the spermathecal eminence might then direct them into the vestibule itself. But, if vaginal currents do exist, two observations suggest they may not be crucial to spermathecal filling. In four cases, the spermathecae were observed to fill *in vitro* when no contractions of the vaginal area were visible and where the common oviducts had been mostly torn away. Contractions of the female genital tract are greatly reduced or abolished at 4° to 7° C. and yet some spermathecal filling can occur. The current produced by the contractions of the lateral oviducts is probably not involved in initially drawing sperm out of the bursa; otherwise sperm should quickly appear in the lower vagina and common oviduct and this occurs only about one hour after spermathecal filling is completed (Spielman, 1964; Jones and Wheeler, 1965). Fifth, the female

might suddenly absorb fluid from one or more thecae into the hemolymph and this would produce a highly specific current. It is difficult to believe, however, that this could occur at 4° to 7° C. The entrance of spermatozoa into the already fluid-filled spermathecae must lead to some displacement of fluid in these organs. While each of the five possibilities referred to might be useful to sperm transport, we have no positive evidence that any one of them is involved in the filling of the spermathecae.

There are some obvious ways in which the activity of the spermatazoa might play an active role in spermathecal filling. First, the headpiece of these cells becomes highly oriented to certain interfaces and is kept in close contact with surfaces (Jones and Wheeler, 1965). Second, the spermatozoa are capable of explosive locomotion and violent spinning whirls, and these movements, together with the thigmotactic head, could account for the sharp U-turn from the bursa into the vestibule. These properties would not, however, explain why the sperm never attempt to enter the open orifice to the accessory gland duct. The speed with which sperm locomote could more than account for their ability to fill the thecae in less than 5 minutes; indeed, the striking thing is that they do not begin to enter the spermathecal ducts as soon as one would expect of such rapidly moving cells.

Artificially injected, active, oriented sperm within the bursa generally are not capable of reaching the thecae by themselves. Even in the normally mated female active sperm stop being transported to the thecae in 5 minutes or less, even though the bursal orifice is wide open and the vestibule is not closed by the ventral tuft (Spielman, 1964; Jones and Wheeler, 1965). That is, something obviously normally inhibits transport very shortly after normal insemination. Whether the female is partially responsible for this is not clear. Gelation and vacuolation of the ejaculate within the bursa cannot be entirely responsible because some sperm can reach the thecae in the absence of vacuolation (as in the cold experiments).

Whatever the explanation of our general failure to achieve good spermathecal filling following artificial insemination, it is certainly not due to failure to distend the bursa fully with numerous highly active spermatozoa capable of rapid locomotion on being released. Neither is it due to the lack of stimulation of the vagina by the aedeagus. Perhaps it is simply a matter of finding the right medium for the sperm but the fact that sperm in fresh ejaculates from donor females do not transfer makes even this seem unlikely.

Before spermathecal filling in *Aedes aegypti* can be understood we shall need to find out (1) what factors are involved in initiating the process once the bursa is inseminated (that is, why do the sperm "wait" before they start to enter the vestibule?), and (2) what factors are responsible for terminating the process after the thecae fill (that is, why do active sperm at the bursal orifice stop entering the vestibule when there is no mechanical blockade and when the third theca is generally never fully filled with sperm?).

A few comparisons may be made between sperm transport in *Aedes* and other insects. According to Nonidez (1920), the sperm of *Drosophila* are deposited initially in the "uterus," and those near the female's accessory gland ducts suddenly become active and swim into the tubular ventral receptacle, which fills in two to five minutes. In *Aedes,* there is no indication that sperm are activated by the female's accessory gland. Although Nonidez stated that after the ventral receptacle

fills, the spermathecae then fill with sperm. DeVries (1964) reported sperm in the "storage organs" of *Drosophila* in less than one minute after coitus. Certain mutants of *Drosophila* which lack both accessory glands (parovaria) and spermathecae can produce viable offspring two to three days after copulation because the tubular receptacle alone can briefly serve as a storage organ (Anderson, 1945). Studies in this laboratory indicate that the sperm of *Aedes* must be stored in the thecae before they are capable of fertilizing the eggs.

Aedes spermatozoa *in vitro* are not obviously chemotactically directed to the vestibule, spermathecae, or their ducts, and thus are quite different from the sperm of the bed bug (Abraham, 1934). Since *Aedes* do not obviously pump sperm into their thecae, they are very different from *Ephestia* and *Plodia* (Norris, 1932) and the honey bee (Ruttner, 1956). The *Aedes* female never transports dead sperm to her thecae and is thus quite unlike the *Rhodnius* female which is said to be capable of doing this (Davey, 1958). The low degree of fertility following artificial insemination of both *Aedes* and *Drosophila* (Gottschewski, 1937) is in striking contrast to the high degree of fertility that is achievable by artificial insemination of the queen honey bee (Laidlaw 1944) and the bed bug (Davis, 1965). Indeed, in the queen honey bee, it is easy to obtain 100% fertility (Laidlaw, personal communication) and the artificially inseminated bed bug lays even more eggs than if she has been naturally mated (Davis, personal communication)!

We are grateful to Drs. Norman T. Davis, Arden O. Lea, P. T. Lum, A. Glenn Richards, Andrew Spielman and Elizabeth D. Jones for valuable suggestions and discussions.

Summary

1. Spermathecal filling in *Aedes aegypti* is not affected by changing the orientation of the intact female's terminalium.

2. Crushing the head of the female before forced-copulation tends to interfere with coitus. Decapitation of the female before forced-mating may lead to prolonged coitus but this does not necessarily result in insemination or spermathecal filling.

3. Crushing the head of the female during the act of forced-copulation does not interfere with coitus, insemination, or spermathecal filling. Crushing of the female's head immediately after coitus does not interfere with spermathecal filling.

4. Spermathecal filling can occur when the freshly inseminated female's terminalium is cut off into a drop of saline.

5. On a few occasions, spermathecae have been seen to fill with sperm in oil-covered saline whole mounts of the isolated reproductive system of a freshly inseminated female.

6. Sperm can ascend to the thecae during the time of oviposition, presumably between the intervals of egg depositions.

7. Spermathecal filling can occur in females which are externally immobilized by exposure to nitrogen, ether, carbon dioxide and cyclopropane.

8. Submerging freshly inseminated females in an ice bath at 4° to 7° C. for 10 to 30 minutes did not prevent spermathecal filling; at these temperatures the sperm are generally quite active. At 1° to 2° C., however, spermathecal filling is inhibited; at such temperatures sperm activity is greatly reduced or abolished.

9. Male *Aedes* can copulate with dead females and may deposit active sperm in the bursa but spermathecal filling does not occur.

10. Dyes injected only into the bursae of virgin females may be transported to one, two, or all three thecae, and to the common oviduct as far as the ampullae. But, dyes injected into the bursae of inseminated females are not transported to the thecae.

11. When seminal vesicle sperm with or without additives was injected into the bursa, only a few sperm reached the large theca in 13 (18.3%) of 71 cases.

12. When fresh ejaculates from donor bursae were injected into recipient virgin bursae, considerably better results were obtained in some cases and the sperm reached one or two thecae in 5 (35.7%) out of 14 cases.

13. The female is incapable of transporting dead sperm to her spermathecae. Highly active and oriented sperm artificially injected into the bursa generally do not fill the spermathecae.

14. The behavior of the sperm alone is not capable of explaining normal spermathecal filling. The role of the female during spermathecal filling is not clear.

LITERATURE CITED

ABRAHAM, R., 1934. Das Verhalten der Spermien der weiblichen Bettwanze (*C. lectularius* L.) und der Verblieb der überschussigen Spermasse. *Zeitschr. Parasitenk.*, **6**: 559–591.

ANDERSON, R. C., 1945. A study of the factors affecting fertility of lozenge females of *Drosophila melanogaster*. *Genetics*, **30**: 280–296.

BISHOP, D. W., 1961. Biology of spermatozoa. *In:* W. C. Young's Sex and Internal Secretions. Volume 2, chapter 13: 707–796.

BISHOP, G. H., 1920. Fertilization in the honey bee. 1. The male sexual organs: their histological structure and physiological functioning. *J. Exp. Biol.*, **31**: 226–265.

BLUNCK, H., 1912. Das Geschlechtsleben des *Dytiscus marginalis* L. 1. Teil. Die Begattung. *Zeitschr. wiss. Zool.*, **102**: 169–248.

BONHAG, P. F., AND J. R. WICK, 1953. The functional anatomy of the male and female reproductive systems of the milkweed bug, *Oncopeltus fasciatus* Dallas (Heteroptera: Lygaeidae). *J. Morph.*, **93**: 177–283.

BURCHAM, E. G., 1957a. Some characteristics and relations of mating and oviposition of *Aedes aegypti* (Linnaeus) (Diptera: Culicidae). Ph.D. Thesis, Ohio State University, 130 pp.

BURCHAM, E. G., 1957b. Artificial insemination of *Aedes aegypti* (L.). *Canad. Ent.*, **89**: 494–495.

CALLAHAN, P. S., AND T. CASCIA, 1963. Histology of the reproductive tracts and transmission of sperm in the corn earworm, *Heliothis zea*. *Ann. Ent. Soc. Amer.*, **56**: 535–556.

CURTIN, T. J., AND J. C. JONES, 1961. The mechanism of ovulation and oviposition in *Aedes aegypti*. *Ann. Ent. Soc. Amer.*, **54**: 298–313.

DAVEY, K. G., 1958. The migration of spermatozoa in the female of *Rhodnius prolixus* Stål. *J. Exp. Biol.*, **35**: 694–701.

DAVIS, N. T., 1965. Studies of the reproductive physiology of Cimicidae (Hemiptera). II. Artificial insemination and the function of the seminal fluid. *J. Ins. Physiol.*, **11**: 355–366.

DEVRIES, J. K., 1964. Insemination and sperm storage in *Drosophila melanogaster*. *Evol.*, **18**: 271–282.

EPHRUSSI, B., AND G. W. BEADLE, 1936. A technique for transplantation for *Drosophila*. *Amer. Nat.*, **70**: 218–225.

GIGLIOLI, M. E. C., 1963. The female reproductive system of *Anopheles gambiae melas*. The structure and function of the genital ducts and associated organs. *Riv. Malariol.*, **42**: 149–176.

GOTTSCHEWSKI, G., 1937. Künstliche Befruchtung bei *Drosophila*. *Naturwiss.*, **25**: 650.

Hewer, H. R., 1934. Studies in *Zygaena*, II. The mechanism of copulation and the passage of sperm in the female. *Proc. Zool. Soc. London*, 513–527.

Hinton, H. E., 1963. Sperm transfer in insects and the evolution of haemocoelic insemination. *Roy. Ent. Soc. London, Symposium*, **2**: 95–107.

Jones, J. C., and R. E. Wheeler, 1965. Studies on spermathecal filling in *Aedes aegypti* (Linnaeus). I. Description. *Biol. Bull.*, **129**: 134–150.

Laidlaw, H. H., 1944. Artificial insemination of the queen bee (*Apis mellifera* L.): morphological basis and results. *J. Morph.*, **74**: 429–465.

Leahy, M. G., 1962. Barriers to hybridization between *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae). Ph.D. thesis, University of Notre Dame, 128 pp.

Ludwig, W., 1926. Untersuchungen über den Copulationsapparat der Baumwanzen. *Zeitschr. Morph. Ökol. Tiere*, **5**: 291–380.

McDaniel, I. N., and W. R. Horsfall, 1957. Induced copulation of aedine mosquitoes. *Science*, **125**: 745.

Nonidez, J. F., 1920. The internal phenomena of reproduction of *Drosophila*. *Biol. Bull.*, **39**: 207–230.

Norris, M. J., 1932. Contributions toward the study of insect fertility. The general structure and operation of the reproductive organs of the genera *Ephestia* and *Plodia* (Lepidoptera, Phycitidae). *Proc. Zool. Soc. London*, 595–611.

Omura, S., 1938. Structure and function of the female genital system of *Bombyx mori* with special reference to fertilization. *J. Fac. Hokkaido Imp. Univ. Sapporo*, **40**: 111–128.

Parkes, A. S., 1960. The biology of spermatozoa and artificial insemination. *In:* F. H. A. Marshall's Physiology of Reproduction. Volume 1, part 2, chapter 9: 161–263. Longman's, Green & Co., Ltd., London.

Richards, A. G., 1963. The rate of sperm locomotion in the cockroach as a function of temperature. *J. Ins. Physiol.*, **9**: 545–549.

Ruttner, F., 1956. Zur Frage der Spermaübertragung bei Beinenkönigin. *Insectes Soc.*, **3**: 351–359.

Schwartz, P. H., 1961. Behavior of spermatozoa in *Aedes aegypti* (L.). Thesis, M.S., University of Maryland, 45 pp.

Snodgrass, R. E., 1956. The Anatomy of the Honey Bee. Comstock Publishing Assoc., Ithaca, xiv + 334 pp.

Spielman, A., 1964. The mechanics of copulation of *Aedes aegypti*. *Biol. Bull.*, **127**: 324–344.

Weidner, H., 1934. Beitrage zur Morphologie und Physiologie des Genitalapparates der weiblichen Lepidopteren. *Zeitschr. angew. Ent.*, **21**: 239–290.

Wheeler, R. E., 1962. A simple apparatus for forced copulation of mosquitoes. *Mosq. News*, **22**: 402–403.

Wheeler, R. E., 1964. Studies on the physiology of hemolymph coagulation in *Periplaneta americana* (L.). Ph.D. Thesis, University of Maryland, 62 pages.

Wheeler, R. E., and J. C. Jones, 1963. A technique for artificial insemination of *Aedes* mosquitoes. *Mosq. News*, **23**: 313–315.

Wigglesworth, V. B., 1953. The Principles of Insect Physiology. E. P. Dutton, N. Y., viii + 546 pp.